AFFINITY PRECIPITATION OF ENZYMES

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1. Introduction

Bifunctional nucleotide derivatives, i.e., nucleotides connected by a spacer, such as AMP–AMP or the heterofunctional compound AMP–ATP [1], have earlier been prepared with the objective of using them primarily as affinity chromatography ligands. It occurred to us that compounds of this type might function as precipitating agents for enzymes. In particular, dimeric NAD-derivatives should be useful since NAD has affinity for a large number of enzymes. Further, these derivatives would allow utilization of the principles of ternary complex formation, which increases the interaction and ensures a high degree of specificity [2]. Such affinity precipitation of enzymes should not only provide a new tool in the analysis and purification of enzymes (dehydrogenases), but also be useful in morphologic and topographic studies of dehydrogenases in analogy to studies using bis-biotinyl diamines and avidin [3].

This paper describes the preparation of a bifunctional NAD compound, N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD) (Bis-NAD), and its properties as a complexing/precipitating agent for the tetrameric enzyme lactate dehydrogenase (LDH).

2. Materials and methods

2.1. *Materials*

LDH (beef heart, type III, in $(NH_4)_2SO_4$, 550 U/mg), adipic acid dihydrazide and 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide were obtained from Sigma, St. Louis, MO. Before use the enzyme was dialyzed overnight against 0.05 M sodium phosphate buffer (pH 7.5) and freed from insoluble matter by centrifugation. N^6-Carboxymethyl-NAD was prepared as in [4]. All other reagents were of analytical grade and obtained from commercial sources.

2.2. *Synthesis of N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD) (Bis-NAD)*

Adipic acid dihydrazide dihydrochloride (105 mg, 0.60 mmol) and N^6-carboxymethyl-NAD (900 mg, 1 mmol) were dissolved in 10 ml water (15°C) and pH adjusted to 4.0 with 1 M HCl. The condensing agent, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, was added as a 1 M aqueous solution (0°C) in 0.1 ml portions. After 30 min by which time 15 portions (1.5 mmol) had been added, thin-layer chromatography indicated that most of the N^6-carboxymethyl-NAD had been converted and the reaction was terminated. The reaction mixture was diluted to 1.0 l and adjusted to pH 8.0 with NH_4OH. The solution was applied to a column with cellulose anion exchanger (Whatman DE-52, 2.5 × 80 cm), successively equilibrated with 1 M NH_4HCO_3 (pH 8.0) and water. The column was washed with water and the nucleotide was then eluted with a 4 l ammonium bicarbonate gradient from 0–0.25 M.

The desired product, Bis-NAD, was eluted between 1.2 l and 1.6 l. Lyophilization gave 400 mg of a white product which was homogeneous as judged from thin-layer chromatography. R_F values on silica gel (Merck, Darmstadt) developed in 0.5 M ammonium acetate : ethanol = 2.5 were: N^6-carboxymethyl-NAD = 0.22; Bis-NAD = 0.05; and the monosubstituted derivative

N_2-adipodihydrazido-N^6-carbonylmethyl-NAD = 0.19. Based on ultraviolet spectra and phosphate analysis the ϵ-value at 266 nm in neutral aqueous solution for Bis-NAD was 21 400 M^{-1} cm^{-1}. The yield based on N^6-carboxymethyl-NAD was calculated as 44%.

2.3. *Affinity precipitation of LDH (standard procedure)*

The precipitation procedure was carried out at 0–6°C in the following way: To 1.5 ml dialyzed LDH (1.1 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.5) was added 0.25 ml 0.12 mM Bis-NAD (water) followed by 0.25 ml 0.8 M sodium pyruvate (water). After gentle mixing in a test tube the solution was allowed to stand. Within 30 min, a heavy precipitate began to form and after 16 h (overnight) the precipitate was isolated by centrifugation.

The amount of LDH in the precipitate and the supernatant fluid was determined by the Lowry procedure [5] and from activity measurements. LDH activity was determined by following the oxidation of NADH at 340 nm. The assay medium consisted of 1 mM sodium pyruvate and 0.30 mM NADH in 0.05 M phosphate buffer (pH 7.5). The assay was initiated by adding a suitably diluted enzyme solution (to give ΔA_{340} <0.1/min). Some enzyme inhibition due to ternary complex formation with Bis-NAD and pyruvate was sometimes observed, but was corrected for by references.

2.4. *Affinity precipitation in agarose gels*

Agarose gels (0.8%) were cast on microscope glass slides and wells were punched out with a die. In double diffusion experiments [6] the agarose gel contained 0.3 M sodium pyruvate and 0.05 M sodium phosphate buffer (pH 7.5); the center well contained 15 μl LDH solution (5–50 μg enzyme) and the peripheral wells contained 7 μl reagent (Bis-NAD, or NAD or buffer). After diffusion for 1.5–16 h in a moist chamber at room temperature the precipitated protein was stained with Amido black [6].

In single radial diffusion experiments [6] the agarose was cast in the presence of pyruvate and Bis-NAD when LDH was to be quantified and in the presence of pyruvate and LDH when Bis-NAD was to be quantified.

3. Results and discussion

3.1. *Structural assignment*

When designing the preparation of bifunctional NAD compounds, principally equivalent to the one depicted in fig.1, i.e., compounds built by connecting two NAD entities symmetrically by a spacer, it appeared reasonable to use the available compounds N^6-carboxymethyl-NAD [4] or N^6-[*N*-(6-aminohexyl)-carbamoylmethyl]-NAD [4]. A direct condensation of these two NAD analogs by a carbodiimide proved unsatisfactory since the yield was very low owing to side reactions [1]. Other methods based on condensation of two N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-NAD with reactive diimidoesters (adipimidate) or with very reactive diacid dichlorides (adipic acid dichloride) were feasible, although the exact procedure when mixing reactants was critical and great care had to be exercised if a satisfactory result was to be expected. In addition, the connecting spacers in these cases were considered unnecessarily long and an alternative linking procedure based on N^6-carboxymethyl derivatives of NAD was therefore tried. N^6-Carboxymethyl-NAD was thus condensed with adipic acid dihydrazide in a carbodiimide-mediated reaction, giving a bis-nucleotide with a spacer of moderate length (fig.1). The distance between the exocyclic nitrogen atoms of the two adenines is ~17 Å (measured from an extended space-filling model), a distance that should allow easy and simultaneous interaction with active sites of two dehydrogenase molecules, provided the sites are not too-deeply buried. The spacer is comparatively hydrophilic, which is preferable because it reduces the risk of non-specific hydrophobic interactions.

The condensation reaction described in section 2 proceeds very smoothly at pH 4.0, due to the favourable pK_a values of the reactants (2.5–3.0 for

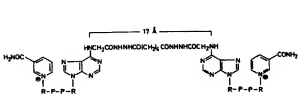

Fig.1. Bis-NAD = N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD).

N^6-carboxymethyl-NAD and 4.9 for adipic acid dihydrazide) which also allow for sufficient buffering to make external pH-control virtually unnecessary.

The structure assigned to Bis-NAD is based on several facts. The method of synthesis, a carbodiimide mediated condensation of N^6-carboxymethyl-NAD and adipic acid dihydrazide, should yield only two new compounds (besides breakdown products), namely adipic acid dihydrazide mono- and disubstituted with NAD. In agreement herewith, an ion-exchange chromatography of the crude reaction product gave three major peaks, the last of which to emerge was unreacted N^6-carboxymethyl-NAD. The other two peaks were assigned to be the monosubstituted compound, N_2-adipodihydrazido-N^6-carbonylmethyl-NAD (the first peak; yield ~10%) and Bis-NAD (the second peak; yield ~40%). In a separate verifying experiment the condensation was carried out with a 20-fold excess of adipic acid dihydrazide and, as expected, only one product was formed, namely the monosubstituted compound. The monosubstituted compound gave positive reaction with trinitrobenzene sulfonic acid reagent [7], whereas Bis-NAD did not, proving that the latter compound lacked the free hydrazide group.

Comparison of 100 MHz proton NMR spectra of N^6-carboxymethyl-NAD, N_2-adipodihydrazido-N^6-carbonylmethyl-NAD and Bis-NAD also confirmed the structures assumed. The two latter compounds thus gave two new signals corresponding to the protons of the four adjacent methylene groups of the spacer. Besides physicochemical tests, the Bis-NAD was shown to act as a coenzyme with LDH.

3.2. *Affinity precipitation, basic properties*

Initial experiments showed that LDH could be precipitated from a solution containing equivalent amounts of Bis-NAD (i.e., 1 NAD/enzyme subunit). The explanation to the precipitation event is believed to be rather straightforward. Molecules of LDH, Bis-NAD and pyruvate form strong dead-end ternary complexes and since Bis-NAD can interact with two LDH molecules and since LDH is a tetrameric enzyme it can easily be understood that large aggregates will form. When these aggregates have grown sufficiently large, they turn insoluble and precipitate out.

In order to obtain an optimal 'yield' of precipitation several parameters were varied. The pH value

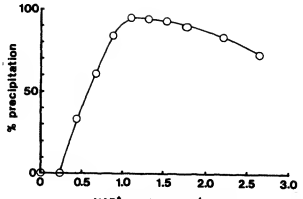

Fig.2. The efficiency of the affinity precipitation as function of the NAD-equiv./subunit ratio. LDH (20 μN) dissolved in 0.05 M phosphate buffer (pH 7.5) 0.1 M with respect to pyruvate was treated with varying amounts of Bis-NAD in small test tubes. Total volume was 0.4 ml. The mixture was kept at 4°C overnight and the precipitated LDH centrifuged down. The amount of precipitated LDH was measured directly by the Lowry method or calculated from the LDH activity of the supernatant.

did not appreciably affect the precipitation in the region pH 6.5–8.0 (phosphate buffer). The concentration of pyruvate was not critical if well above the mM range. Therefore 0.1 M pyruvate was routinely employed.

The correct stoichiometry of reactants (Bis-NAD/enzyme) in the affinity precipitation process is obviously important. Figure 2 gives the degree of precipitation as a function of the ratio between NAD-equivalents and enzyme subunits. It is evident that maximum precipitation occurs near equinormality of enzyme subunits and NAD. When the ratio between coenzyme equivalents and enzyme subunits is lower than unity, the precipitation yield is low, e.g., a ratio of 0.3 gives hardly any precipitation. On the other hand, if the ratio is above unity, the precipitation yield is not so markedly affected, a ratio of 2.5, for example, still giving a precipitation efficiency of 75%.

To confirm these results an additional experiment was carried out in which the composition of the precipitate was determined. For this purpose a precipitate prepared by the standard procedure was centrifuged and treated with urea to obtain a clear

solution. The solution was analyzed in ultraviolet light (266 nm and 290 nm) and the content of nucleotide and enzyme calculated. It turned out that the composition of the precipitate corresponded well to the ratio at which the precipitation was best, i.e., 1.1 NAD equiv./LDH subunit.

To obtain macromolecular aggregates, a minimum of 2 (average) subunits/LDH molecule must be engaged in complexes, and this minimum corresponds to a ratio of 0.5; when all subunits are engaged the ratio would be 1.0, a value close to the observed value of 1.1. This reasoning is, of course, valid only with the assumption that the cofactor, pyruvate and enzyme form a firm complex. It is also assumed that Bis-NAD does not participate in intramolecular crosslinking, an unlikely situation since the 17 Å spacer (fig.1) would be too short to cover the distance between two cofactor binding sites within the same molecule [8].

3.3. *Redissolution of affinity precipitated dehydrogenase*

In some applications of affinity precipitation, e.g., purification of enzymes, it would be necessary to remove pyruvate and Bis-NAD as a final step, and in such a way as to preserve activity. Table 1 illustrates the feasibility of regenerating the free enzyme from the precipitate by gel filtration. In order to discriminate between losses of activity due to the affinity precipitation process and losses due to the regeneration, several references were included. Gel filtration on Sephadex G-50 gave a recovery of ~85%, the 15% loss of activity being caused both by the precipitation and the gel filtration, as judged from the references. Prior to the gel filtration the precipitate had to be dissolved and to this end NADH was added to 10 mM. The precipitate dissolved within 1 min, the mechanism behind the phenomenon obviously being that NADH forms a strong complex with LDH (K_{diss} ~1 μM, NADH is present in high concentration) and thus efficiently competes for the active sites. Also, the simultaneous presence of NADH and LDH would consume the comparatively small amount of pyruvate present in the precipitate. Another competitive ligand, AMP (10 mM) was also tried, but was less efficient in solubilizing the precipitate in accordance with its higher dissociation constant, K_{diss} ~1 mM. Summarizing, it is feasible by simple means to regenerate affinity precipitated material.

3.4. *Affinity precipitation in gels*

Affinity precipitation of enzymes resembles, at least superficially, immunoprecipitation, i.e., the aggregation of antibodies and antigens. In a set of experiments we exploited this and adopted the techniques commonly used in immunodiffusion

Table 1
Recovery of affinity precipitated LDH

Sample reference	Additions in the precipitation step	Treatment in the recovery step	Recovery %
Sample	Pyruvate + Bis-NAD	NADH; G-50	85
Reference 1	Pyruvate + NAD	NADH; G-50	92
Reference 2	None	NADH; G-50	92
Reference 3	None	None	100

Samples and references contained 25 μN LDH in 0.05 M phosphate buffer (pH 7.5). In the precipitation step pyruvate (0.1 M) and Bis-NAD (30 μN) or NAD (30 μN) were added as indicated. The total volume was 1.20 ml. After 16 h the precipitate (formed in the samples only) was centrifuged down and the supernatant discarded. In the recovery step the LDH in samples and references was freed from pyruvate and nucleotide by gel filtration (Sephadex G-50, 1.5 × 30 cm; 1 ml/min). Before application on the G-50 column the sample and the reference were steeped in 10 mM NADH (in order to dissolve the precipitated protein in the sample). The activity of the LDH after the various treatments is given relative to that of reference 4 (100%)

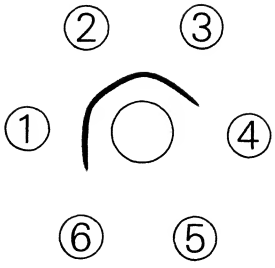

Fig.3. Diffusion–precipitation in agarose gel. The center well contained 30 μg lactate dehydrogenase in 15 μl buffer. The peripheral wells contained 7 μl reagents: wells 1–3, 1.5 nmol Bis-NAD; well 5, 3.0 nmol NAD; wells, 4, 6, buffer only.

experiments. Figure 3 shows the result of a double diffusion experiment (Ouchterlony test [6]) in agarose-containing pyruvate, where the center well contained enzyme, the peripheral wells Bis-NAD or NAD or buffer. A precipitation band was observed only where LDH and Bis-NAD had been allowed to diffuse towards each other. The figure shows the situation after 1.5 h diffusion. Prolonged diffusion for 16 h gave approximately the same pattern. The single radial diffusion technique (Mancini method [6]) was briefly evaluated with respect to its ability to quantify lactate dehydrogenase and/or Bis-NAD. The agarose gel in this case was cast in the presence of pyruvate and Bis-NAD (or lactate dehydrogenase) and the linearily-arranged wells were filled with different amounts of lactate dehydrogenase (or Bis-NAD). Precipitation rings were formed around the wells; the diameters being approximately proportional to the amount of lactate dehydrogenase (or Bis-NAD) present in the well.

4. Conclusion

Affinity precipitation of enzymes has been exemplified here with lactate dehydrogenase and Bis-NAD (+ pyruvate) and is likely to be applicable also to other NAD-dependent dehydrogenases although preliminary experiments with liver alcohol dehydrogenase and the Bis-NAD used in this study (+ pyrazol) indicate that at least for alcohol dehydrogenase a longer spacer is required. Affinity precipitation should be applicable also to enzymes/proteins other than dehydrogenases, provided that suitable bifunctional ligands are available. In cases in which the interaction between the bifunctional ligand and the enzyme is not sufficiently strong, formation of ternary complexes could be tried as exemplified here. Thus, besides dead-end complex formation also other ternary complexes could be utilized, for instance complexes with coenzyme and inhibitor and complexes with coenzyme–substrate adducts [9]. Alternatively, a careful addition of salts, e.g., ammonium sulphate or solvents, e.g., polyethylene glycol might enhance the precipitation without impairing the specificity.

Affinity precipitation of enzymes using bis-nucleotides of varying spacer length may be useful in topographic studies of enzymes, e.g., in the determination of the depth of an active site/ligand binding site. Also information concerning the spatial arrangements of subunits in an oligomeric enzyme might be obtained from precipitated aggregates by using electron microscopy analogous to the study of complexes between avidin and bisbiotinyl diamines [3]. In certain cases enzyme purification procedures may benefit from the principle, e.g., when conventional affinity chromatography is less satisfactory because of sterically-impeded interaction between the enzyme and its immobilized ligand. Further, the method of diffusion–precipitation in gels might find, for example, clinical applications. The procedure could, for instance, be used for detecting abnormal levels of enzymes and other proteins found in serum; compared with the immunodiffusion technique an obvious advantage of this method is that antibodies, which might be difficult to raise, would not be needed.

Finally, it deserves mentioning that bifunctional ligands (including those interacting with effector sites) may prove useful as agents permitting precipitation/immobilization of biomolecules in a reversible manner.

Acknowledgement

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